WRAIR INVENTION DISCLOSURE SUMMARY

(To be attached to all Invention Disclosures submitted by WRAIR inventors; Type; Do not exceed one page)

WRAIR Invention Docket No.: WR 95112 Log 95-16

Date of Invention Disclosure: June 30, 1995

Invention Disclosure Title: Construction of an attenuated Shigella flexneri 2a strain 2457T for use as a DNA delivery vehicle for DNA-mediated immunizations and for a potential vaccine candidate against Shigella flexneri infection.

Inventor(s) and WRAIR Division or other Affiliation:

Arthur A. Branstrom, Department of Bacterial Diseases, Division of Communicable Diseases and Immunology.
Donata R. Sizemore, Department of Bacterial Diseases, Division of Communicable Diseases and Immunology.
Jerald C. Sadoff, Division of Communicable Diseases and Immunology.

1. Narrative Description of the Invention [Highlight what the Invention does and how it differs from conventional practices; identify other parties (especially commercial interests) to this invention, and state whether this is a Subject Invention under a CRDA, contract, grant, or other form of Agreement]:

The invention is an *asd* attenuated isolate of *Shigella flexneri* 2a strain 2457T. This strain has been mutated in the gene encoding aspartate β-semialdehyde dehydrogenase (ASD). This mutation results in a strain unable to grow in the absence of diaminopimelate (DAP), an essential peptidoglycan component comprising the cell wall of gram negative bacteria. DAP is not present in mammalian tissues, and is therefore unavailable for scavenge by infecting bacteria. This strain will likely be a better carrier of DNA for DNA-mediated immunizations. At present, there are no strains of *Shigella* that are sufficiently attenuated to not cause disease, still maintain the capacity to invade mammalian cells, and then quickly die once inside the host cell. We believe the 15D strain meets these requirements, and has already been shown to be an effective vehicle for delivering DNA to BHK and P815 cells grown in culture. Secondly, the construction of an *asd* attenuated *S. flexneri* strain may serve as a potential vaccine candidate for preventing *S. flexneri* disease. Current attenuating mutations in *Shigella* have failed to result in the development of an effective vaccine against *Shigella* infection. The *asd* mutation will likely be safer compared with other attenuating components, since mutating the *asd* gene creates a bacteria which cannot divide and subsequently dies in the absence of DAP. We have already demonstrated the successful attenuation of 15D, and its ability to protect against a challenge in the guinea pig keratoconjuctivitis model. We believe this strain may have the capability of eliciting an immune response which will protect individuals from disease.

2. Value of the Invention to the U.S. Government and the U.S. Army [Highlight how the invention helps or will help the soldier]:

The *asd* attenuated isolate of *S. flexneri* 2a strain 2457T can be marketed as a bacterial vector for delivery of plasmid DNA for DNA-mediated immunizations. This vaccination approach would be applicable to both government and commercial interests. In addition, this engineered bacteria could serve as a potential vaccine candidate strain against *S. flexneri* infections for military personnel deployed to endemic areas.

3. Commercial Applications of the Invention [Describe to the best of your knowledge the potential for commercialization of the invention, including potential candidates for licensing of the invention; identify any commercial concerns who have approached you concerning this invention; CAUTION: do not approach commercial interests independently]:

The 15D strain would also be a commercial benefit for vaccinating populations of third world countries and people traveling abroad. The strain has the potential for being a carrier of a broad range of immunizing antigens encoded on non-replicating DNA for the purpose of DNA-mediated immunizations. Direct DNA-mediated immunization is an evolving new approach to vaccine development, where DNA encoding foreign proteins is injected directly into the muscle or skin, taken up, then transcribed and translated into products which stimulate the immune system. The technique has relied upon the direct administration of purified bacterial plasmids by injection or transfection on gold particles. We constructed what we believe is a highly attenuated bacterial vector, which is capable of invading mammalian cells. We have shown this strain then breaks out of the phagocytic vacuole, ruptures due to the inability to synthesize DAP, and successfully delivers functional foreign DNA to mammalian cells in culture. This opens the possibility of using this strain for oral and other mucosal DNA immunization and gene therapy strategies. We have shown in an animal model (guinea pig keratoconjunctivitis) 15D fails to cause disease and protects from a challenge with virulent 2457T.

Exhibit 7

DEC- 1-1995 03:04PM FROM-FITZSIMONS ADVISOR

UNITED STATES OF AMERICA

INVENTION DISCLOSURE

(THIS FORM AND ACCOMPANYING DRAWING AND DESCRIPTION SHEETS ARE TO BE COMPLETED FOR EACH INVENTION PROMPTLY FORWARDED TO THE PATENT ACTIVITY(S))

PATENT ACTIVITIES | DOCKET NO.

ASSIGNED TO:

SHORT TITLE OF INVENTION: Construction of an attenuated *Shigella flexneri* 2a strain 2457T for use as a DNA delivery vehicle for DNA-mediated immunizations and for a potential vaccine candidate against *Shigella flexneri* infection.

FULL NAME(S) OF INVENTOR(S) (FIRST) (MIDDLE INITIAL) (LAST)	HOME ADDRESS(ES)	(DUTY) TEL. NO. AREA CODE
(1) Arthur A. Branstrom	309 Cedar Lane, Rockville MD 20851	(202)782-3013
(1) ~~Donata R. Sizemore~~	~~10008 Sedwick Rd. #301, Gaithersburg MD 20879~~	(202)782-3013
(2) Jerald C. Sadoff	1622 Kalmia Rd. NW, Washington DC 20012	(610)277-4639
(3)		

INFORMATION AND DATES CONCERNING THIS INVENTION

NEEDED IN THE EVENT OF A CONTEST OF PRIORITY OF INVENTION OR THE U.S. PATENT AND TRADEMARK OFFICE. ALL RECORDS CITED SHOULD BE DATED AND SIGNED BY TWO INDEPENDENT WITNESSES WHO HAVE READ AND UNDERSTOOD THE MATERIAL.

ON WHAT DATE DID YOU FIRST THINK OF THIS INVENTION (WHAT RECORDS SHOW THIS?)
(4) Primers for *asd* deletion were designed and synthesized 08/12/93.

GIVE DATE OF AND IDENTIFY EARLIEST SKETCH OR DRAWING
(5) Freezer stock of constructed suicide vector for chromosomal mutagenesis of *asd* made 01/19/94. The mutated *S. flexneri* was made and officially documented 09/19/94, Laboratory Notebook #A0620

WHEN/WHERE AND TO WHOM DID YOU MAKE THE FIRST DISCLOSURE TO OTHERS OF THE INVENTION EITHER ORALLY OR IN WRITING?
(6) June, 1993/Palo Alto. Orally and in writing (under confidentiality) to Dr. Nelson Teng.

DESCRIBE DETAILS OF ANY WORK OR TESTS DONE TO PRODUCE OR OPERATE THE INVENTION GIVE DATES AND WITNESSES (USE OTHER PAGES IF NECESSARY)
(7) See attached documentation for item 14A (Drawing and Description Sheet)

DESCRIBE AND GIVE DATES OF ANY OTHER SKETCHES, DRAWINGS OR REPORTS PERTINENT TO THIS INVENTION
(8) June 1993, at Palo Alto, discussed in confidence with Dr. Nelson Teng.

USE, SALE OR PUBLICATION

NEEDED TO ESTABLISH THE DATE OF ANY PRINTED PUBLICATION, PUBLIC USE OR SALE. SINCE NO PATENT APPLICATION MAY BE FILED AFTER ONE YEAR FROM SUCH DATE.

IF INVENTION HAS BEEN SOLD OR USED FOR PROFIT - WHEN AND TO WHOM DISCLOSED OR WHEN AND HOW USED?
(9) N/A

HAS A DESCRIPTION OF THIS INVENTION BEEN MADE AVAILABLE TO PERSONS OUTSIDE THE ARMY? (WRITTEN OR ORAL) IF SO, HOW AND WHEN AND WAS USE RESTRICTED?
(10) Yes: Submitted for review for publication on June 7, 1995.
Access made available to Dr. David Hone for purpose of collaboration.

POTENTIAL MARKET INFORMATION

NEEDED FOR POSSIBLE MARKETING INVESTIGATIONS AND AS AN AID TO POTENTIAL LICENSING TO OTHERS.

DESCRIBE ANY POTENTIAL OR EXISTING MARKET FOR SALE OR LICENSE OF THIS INVENTION
(11)
A. GOVERNMENT:
See attached for item #11 A and B.
B. COMMERCIAL:
C. IDENTIFY ANY KNOWN FIRMS OR VENDORS WHO MAY BE INTERESTED IN THE INVENTION

CONTRACT INFORMATION

A DETERMINATION OF RIGHTS IN THIS INVENTION WILL BE NECESSARY. (SEE AR 27-60)

IF THIS INVENTION WAS FIRST CONCEIVED OR CONSTRUCTED IN CONNECTION WITH:
(12)
A. MY DUTIES AS A GOVERNMENT EMPLOYEE
B. MY WORK UNRELATED TO MY DUTIES AS A GOVERNMENT EMPLOYEE (PRIVATE, OFF DUTY ACTIVITIES)
C. MY DUTIES AS A GOVERNMENT EMPLOYEE & WORKING WITH A CONTRACTOR
D. NEITHER A, B OR C. EXPLAIN
Invention conceived outside of job description guidelines, but following the mission goal of vaccine development.

FOREIGN FILING CONSIDERATION

NEEDED TO DETERMINE THE POTENTIAL WORLDWIDE USE FOR THE INVENTION.

INDICATE THE POTENTIAL FOR USING THIS INVENTION IN FOREIGN COUNTRIES
(13)
☐ POOR
☐ GOOD
☒ EXCELLENT

SECURITY CLASSIFICATION

PLEASE INDICATE THE SECURITY CLASSIFICATION IF KNOWN
(13A)
☐ CLASSIFIED
LEVEL
☐ UNCLASSIFIED
☐ CLASSIFICATION UNKNOWN

rovide the following information concerning the disclosed invention and in the indicated sequence:
Specifically describe the invention and its operation. You may use and attach copies of sketches, prints, photographs, papers, and illustrations, which should be signed, witnessed and dated. Use numbers and descriptive names in descriptions and drawings.
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Discuss the problems which the invention is designed to solve, referring to any prior invention of a similar nature with which you may be familiar.
List all known and other possible uses for the invention.
List the features of the invention that are believed to be novel.
USE AS MANY OF THESE SHEETS AS NECESSARY AND ATTACH TO COMPLETED INVENTION DISCLOSURE

11. Potential Market Information:

A and B: The *asd* attenuated isolate of *S. flexneri* 2a strain 2457T can be marketed as a bacterial vector for delivery of plasmid DNA for DNA-mediated immunizations. This vaccination approach would be applicable to both government and commercial interests. In addition, this engineered bacteria could serve as a potential vaccine candidate strain against *S. flexneri* infections for military personnel deployed to endemic areas. This strain would also be a commercial benefit for vaccinating populations of third world countries and people traveling abroad.

14 A. The invention is an *asd* attenuated isolate of *Shigella flexneri* 2a strain 2457T. This strain has been mutated in the gene encoding aspartate β-semialdehyde dehydrogenase (ASD). This mutation results in a strain unable to grow in the absence of diaminopimelate (DAP), an essential peptidoglycan component comprising the cell wall of gram negative bacteria. DAP is not present in mammalian tissues, and is therefore unavailable for scavenge by infecting bacteria. *Shigella flexneri* 2a strain 2457T was mutated by integration of a deleted *E. coli asd* fragment into the chromosome (see figure 1). To accomplish this, the gene encoding *E. coli asd* (1) was amplified using the Polymerase Chain Reaction (PCR), incorporating *BglII* restriction sites. The *asd* PCR product was cloned into a previously described vector (2), which contains a pUC18 backbone and the pSC101 origin of replication. Positive constructs were selected for their ability to complement the *asd E. coli* mutant, χ6097 (3). The resulting pAB102 plasmid was reverse PCR amplified to delete 553 bp of the *E. coli asd* structural gene (position 439 to 991)[all primers given in a 5' to 3' orientation]. The kanamycin resistance cassette from the commercial plasmid pUC4K-KIXX (Pharmacia) was purified as a *SmaI* fragment and cloned between the flanking *asd* sequences. Using forward and reverse primers containing restriction sites *SacI* and *SalI*, respectively, PCR amplification resulted in a 2 kb PCR fragment comprising the *asd* flanking sequences with the internal Kan^r cassette. The entire Δ*asd*::Kan^r PCR fragment was cloned into the *SacI/SalI* site of the positive selection suicide vector pCVD442 (4). Ligations were transformed into strain SM10λpir (5) and selected for resistance to ampicillin. SM10λpir (pCVD442::*asd*) was conjugated with *S. flexneri* 2a strain 2457T (pAB322[Tet^r,Amp^s]) and Amp^r/Tet^r conjugants selected. PCR analysis of chromosomal integrates showed the recombination event occurred in the downstream portion of the cloned *asd* inserted into the pCVD442 plasmid. The integrated plasmid and the intact *asd* were resolved by growing isolates on sucrose containing media, which resulted in a second recombination event (6). Screening for Kan^r and a requirement for DAP, isolate 15C was obtained. Hybridization and PCR analysis confirmed this strain as having a deletion in *asd*. This mutation could be complemented with *E. coli asd* cloned in a low copy number vector (pSC101 origin of replication). 15C was then cured of its Tet^r plasmid by fusaric acid treatment (7) to generate isolate 15D.

See figure 1 on the following page.

TURE(S) AND ORGANIZATION OF INVENTORS(S) (USE INK) | DATE | THE DESCRIBED INVENTION HAS BEEN WITNESSED, READ AND UNDERSTOOD BY: | DATE:

[signature] | 30 June 95 (18) | [signature] | 30 June 95

ORGANIZATION WRAIR

[signature] | 30 June 95 (19) | [signature] | 30 June 95

ORGANIZATION

[signature] | 5 July 95 (20) | [signature] | 5 July 95

ORGANIZATION

E: THIS FORM AND ANY OMITTED INFORMATION BECOMING AVAILABLE AT A LATER TIME SHOULD BE FORWARDED TO:
CHIEF, INTELLECTUAL PROPERTY DIV. DARCOM ATTN: PATENT COUNSEL; OR CHIEF OF ENGINEERS ATTN: PATENT COUNSEL
OFFICE OF THE JUDGE ADVOCATE GENERAL
DEPT. OF THE ARMY
WASHINGTON, D.C. 20310

DRM 3734-R(1 Oct 1978)

INVENTION DISCLOSURE
(DRAWING AND DESCRIPTION SHEET)

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Specifically describe the invention and its operation. You may use and attach copies of sketches, prints, photographs, papers, and illustrations, which should be signed, witnessed and dated. Use numbers and descriptive names in descriptions and drawings.

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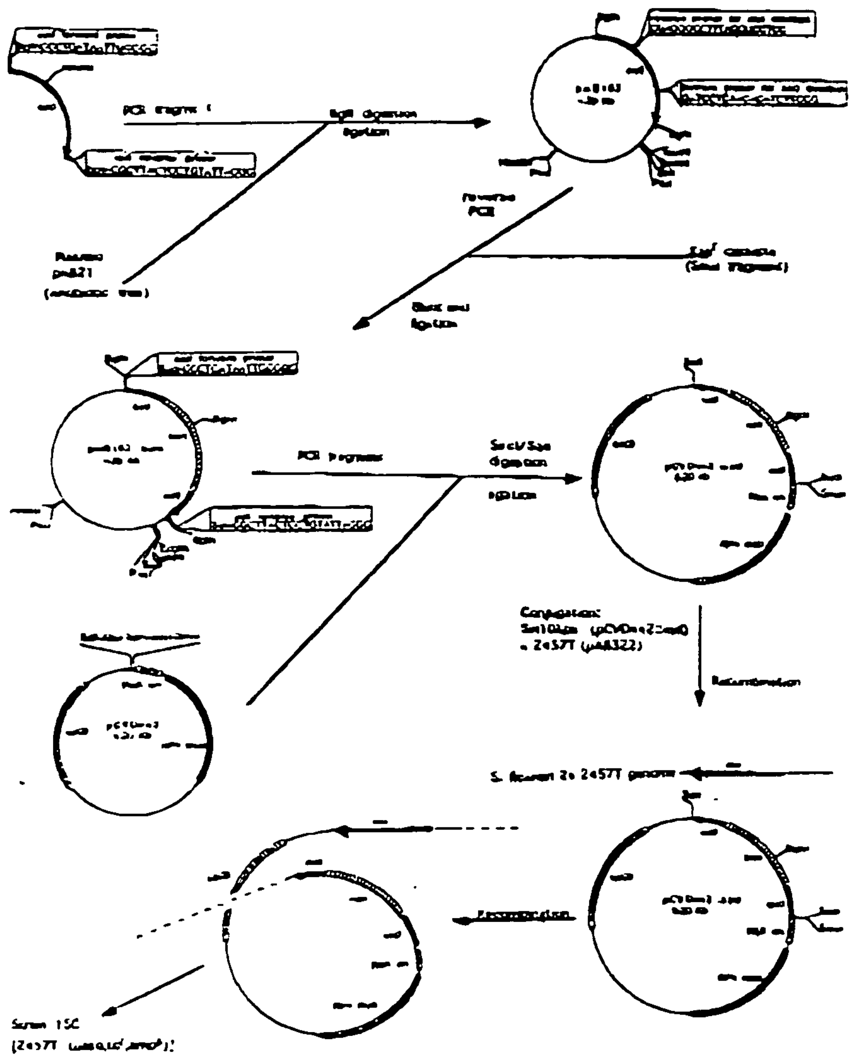
USE AS MANY OF THESE SHEETS AS NECESSARY AND ATTACH TO COMPLETED INVENTION DISCLOSURE

Figure 1: Construction of a Δasd derivative of *Shigella flexneri* 2a strain 2457T.



URE(S) AND ORGANIZATION OF INVENTORS(S) (USE INK) | DATE: | THE DESCRIBED INVENTION HAS BEEN WITNESSED, READ AND UNDERSTOOD BY | DATE

Samuel C Ardoff | 30 June 95 | (18) D Rizzo Gillon | 30 June 95

ORGANIZATION WRAIR

Arthur A Branstein | 30 June 95 | (19) [signature] | 30 June 95

ORGANIZATION

Donata R Sizemore | 5 July 95 | (20) Richard C Wine | 5 July 95

ORGANIZATION WRAIR

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Direct DNA-mediated immunization is an evolving new approach to vaccine development, where DNA encoding foreign proteins is injected directly into the muscle or skin, taken up, then transcribed and translated into products which stimulate the immune system. The technique has relied upon the direct administration of purified bacterial plasmids by injection or transfection on gold particles (8). We set out to construct a *S. flexneri* strain that would serve as a carrier to deliver this immunizing DNA to the cytoplasm of target cells. The operation of the invention centers around the characteristics of the *asd* mutation in *S. flexneri*. We constructed what we believe is a highly attenuated bacterial vector, which is capable of invading mammalian cells. We have shown this strain then breaks out of the phagocytic vacuole, ruptures due to the inability to synthesize DAP, and successfully delivers functional foreign DNA to mammalian cells in culture (9). This opens the possibility of using this strain for oral and other mucosal DNA immunization and gene therapy strategies. We have shown in an animal model (guinea pig keratoconjunctivitis) 15D fails to cause disease and protects from a challenge with virulent 2457T.

14 B. The advantage of using an *asd* attenuated isolate over other attenuated strains of *S. flexneri* is 15D's inability to replicate in the absence of DAP. We believe that the *asd* attenuating feature of 15D will make it a better candidate to serve as a carrier for DNA-mediated immunizations and also as a vaccine candidate. As a potential vaccine candidate, this strain has been shown to be attenuated and protective in a guinea pig keratoconjuctivitis animal model. Previously constructed *Shigella* vaccine candidates have either not elicited a protective immune response to protect against subsequent challenge, or the strains weren't sufficiently attenuated for use in humans.

SIGNATURE(S) AND ORGANIZATION OF INVENTORS(S) (USE INK)	DATE	THE DESCRIBED INVENTION HAS BEEN WITNESSED, READ AND UNDERSTOOD BY:	DATE:
[signature]	30 June 95 (18)	[signature]	30 June 95
ORGANIZATION WRAIR			
[signature]	30 June 95	[signature]	30 JUNE 95
ORGANIZATION			
[signature]	5 July 95 (20)	[signature]	5 July 95
ORGANIZATION WRAIR			

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D. List all known and other possible uses for the invention.
E. List the features of the invention that are believed to be novel.

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14 C. The construction of an isolate of *S. flexneri* containing a deletion in the *asd* gene will potentially solve two problems. First, this strain will likely be a better carrier of DNA for DNA-mediated immunizations. At present, there are no strains of *Shigella* that are sufficiently attenuated to not cause disease, still maintain the capacity to invade mammalian cells, and then quickly die once inside the host cell. We believe the 15D strain meets these requirements, and has already been shown to be an effective vehicle for delivering DNA to BHK and P815 cells grown in culture (9). Secondly, the construction of an *asd* attenuated *S. flexneri* strain may serve as a potential vaccine candidate for preventing *S. flexneri* disease. Current attenuating mutations in *Shigella* have failed to result in the development of an effective vaccine against *Shigella* infection. The *asd* mutation will likely be safer compared with other attenuating components, since mutating the *asd* gene creates a bacteria which cannot divide and subsequently dies in the absence of DAP. We have already demonstrated the successful attenuation of 15D, and its ability to protect against a challenge in the guinea pig keratoconjuctivitis model. We believe this strain may have the capability of eliciting an immune response which will protect individuals from disease.

14 D. This invention has the following potential uses. The strain can successfully serve as a carrier for the delivery of DNA to colonic mucosa, thus opening the possibility of oral and other mucosal DNA immunization and gene therapy strategies utilizing strain 15D. Genes encoding antigens from organisms causing: (a) diarrheal diseases such as rotavirus; (b) sexually transmitted diseases such as human immunodeficiency virus, *Neisseria gonorrhoeae*, and human papilloma virus; and (c) gastrointestinal diseases such as the ulcer causing *Helicobacter pylori* , can be cloned into nonreplicating plasmids. These plasmids can then be carried by 15D for mucosal immunizations. 15D has been found to maintain many different plasmid types without antibiotic selection. Delivery of DNA encoded antigens to the mucosal immune system by strain 15D may permit mucosal immunization simultaneously with multiple antigens that can be directed for class I and/or II presentation, stimulation of Th1 or Th2 help, or secreted maintaining the proper folding and conformational epitopes for IgA and IgG antibody production. While we have constructed a novel strain for delivering functional DNA to the cytoplasm of mammalian cells, this mutation should not be restricted to *Shigella* species, since the invasion genes that *Shigella* utilize can be inserted into other bacteria such as *E. coli* (10). Another potential use of strain 15D is for vaccination against *S. flexneri* infections. This strain should provide for a safe oral vaccine candidate that may have the capacity of eliciting a protective immune response. While determination of the safety of this strain awaits human trials, this *asd* mutation can be applied to other *Shigella* serotypes as an effective attenuator for constructing additional vaccine candidates, both as DNA carriers, and as live-attenuated bacterial vaccines.

SIGNATURE(S) AND ORGANIZATION OF INVENTOR(S) (USE INK)	DATE:	THE DESCRIBED INVENTION HAS BEEN WITNESSED, READ AND UNDERSTOOD BY:	DATE:
[signature] ORGANIZATION WRAIR	30 June 95 (18)	[signature]	30 June 95
Arthur A. Brinster	30 June 95 (19)	[signature]	30 JUNE 95
ORGANIZATION			
Donata R. Sizemore	5 July 95 (20)	[signature]	5 July 95
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14 E. The novel feature of this invention is the use of an *asd* lethal mutation to attenuate a strain of *S. flexneri*. The ASD deficiency results in what we believe will be a sufficiently attenuated isolate that can serve as a delivery vehicle for plasmid DNA and as a potential vaccine candidate against *S. flexneri* infection. It was not predictable and highly unexpected that a strain containing a mutation which does not permit even a single replication is able to invade, deliver DNA, and immunize against itself.

References:

1. C. Haziza, P. Stragier, J.C. Patte, *EMBO J.* 1, 379 (1982).
2. A. Branstrom, D. Sizemore, R. Warren, J. Sadoff. Presented at the 33rd ICAAC, New Orleans, LA, 20 October, 1993, #1136.
3. K. Nakayama, S.M. Kelly, R. Curtiss III, *BioTechnology* 6, 693 (1988).
4. M.S. Donnenberg and J.B. Kaper, *Infect. Immun.* 59, 4310 (1991).
5. R. Simon, U. Priefer, A. Puhler, *BioTechnol.* 1,784 (1983).
6. J. Quandt and M.F. Hynes, *Gene* 127, 15 (1993).
7. S.R. Maloy and W.D. Nunn, *J. Bacteriol.* 145, 1110 (1981).
8. J.J. Donnelly, J.B. Ulmer, M.A. Liu, *J. Immunol. Methods* 176, 145 (1994).
9. D. Sizemore, A. Branstrom, J. Sadoff. Submitted June 7 for publication in *Science*(1995).
10. P.J. Sansonetti, *et al.*, *Infect. Immun.* 39, 1392 (1983).

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[signature]	6/30/95	(19) [signature]	30 JUNE 95
ORGANIZATION WRAIR			
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